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Rolfs, A ; Kvietikova, I ; Gassmann, M ; Wenger, R H

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# Oxygen-regulated Transferrin Expression Is Mediated by Hypoxia-inducible Factor-1\*

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**Transferrin (Tf) is a liver-derived iron transport protein whose plasma concentration increases following exposure to hypoxia. Here, we present a cell culture model capable of expressing Tf mRNA in an oxygen-dependent manner. A 4-kilobase pair Tf promoter/enhancer fragment as well as the 300-base pair liver-specific Tf enhancer alone conveyed hypoxia responsiveness to a heterologous reporter gene construct in hepatoma but not HeLa cells. Within this enhancer, a 32-base pair hypoxia-responsive element was identified, which contained two hypoxia-inducible factor-1 (HIF-1) binding sites (HBSs). Mutation analysis showed that both HBSs function as oxygen-regulated enhancers in Tf-expressing as well as in non-Tf-expressing cell lines. Mutation of both HBSs was necessary to completely abolish hypoxic reporter gene activation. Transient co-expression of the two HIF-1 subunits HIF-1 $\alpha$  and aryl hydrocarbon receptor nuclear translocator (ARNT)/HIF-1 $\beta$  resulted in enhanced reporter gene expression even under normoxic conditions. Overexpression of a dominant-negative ARNT/HIF-1 $\beta$  mutant reduced hypoxic activation. DNA binding studies using nuclear extracts from the mouse hepatoma cell line Hepa1 and the ARNT/HIF-1 $\beta$ -deficient subline Hepa1C4, as well as antibodies raised against HIF-1 $\alpha$  and ARNT/HIF-1 $\beta$  confirmed that HIF-1 binds the Tf HBSs. Mutation analysis and competition experiments suggested that the 5' HBS was more efficient in binding HIF-1 than the 3' HBS. Finally, hypoxic induction of endogenous Tf mRNA was abrogated in Hepa1C4 cells, confirming that HIF-1 confers oxygen regulation of Tf gene expression by binding to the two HBSs present in the Tf enhancer.**

Iron is an essential trace metal in all living organisms. Both iron overload and iron depletion can severely affect physiological processes such as development, erythropoiesis, or biochemical metabolism (reviewed in Refs. 1 and 2). The liver represents the major organ of iron storage in the body and is most susceptible to injuries due to iron overload (1). Thus, iron homeostasis has to be tightly balanced, and, as a consequence, free iron occurs only transiently in the serum. When iron is absorbed from the small intestine into the blood, it immediately

binds apotransferrin to form transferrin (Tf)<sup>1</sup>, which is then transported by the plasma to all tissues of the vertebrate's body. Delivery of iron occurs by binding of Tf to the Tf receptor followed by endocytosis. In erythroblasts, iron is primarily required for heme synthesis in mitochondria. Tissue-specific expression of the Tf gene is controlled by distinct positive and negative regulatory elements located 5' to the transcription initiation site. Apart from the promoter, the best studied element within this region is the -3600/-3300 enhancer (hereafter referred to as the Tf enhancer). This *cis*-acting element enhances the activity of the Tf promoter in human Hep3B hepatoma cells in a tissue-specific manner (3, 4). Studies in Hep3B and HeLa cells revealed that multiple liver-enriched and ubiquitous factors interact with the Tf enhancer (3, 5, 6). The Tf enhancer, however, is inactive in Tf-expressing neuronal and Sertoli cells (4, 5).

Hypoxia, a reduction in oxygen concentration, is increasingly recognized as an important regulator of gene expression (reviewed in Ref. 7). The best established example of oxygen-regulated gene expression is provided by the erythropoietic growth factor erythropoietin (Epo, reviewed in Ref. 8). The two human hepatoma cell lines HepG2 and Hep3B are so far the only permanent cell culture models available to investigate oxygen-regulated Epo expression (9). Apart from Epo, we recently demonstrated hypoxic induction of several acute phase genes in HepG2 cells (10). Acute phase reactants are liver-derived serum proteins whose production is induced by proinflammatory cytokines (reviewed in Ref. 11). Tf expression was of particular interest since this protein is one of the rare examples of acute phase reactants that are down-regulated during the acute phase response in both human serum and HepG2 cells (11). In contrast, we found a marked increase in Tf transcription following hypoxic (1% O<sub>2</sub>) culture of HepG2 cells (10), suggesting that different signaling pathways are mediating the effects of these two stimuli. Given that the erythroid marrow uses more than 80% of plasma iron (2), and considering that hypoxia increases erythropoiesis, it is conceivable that an increase in plasma iron transport capacity is required for hypoxia-induced Epo-mediated erythropoiesis. Indeed, hypoxia was shown to increase iron absorption (12), and hypoxic up-regulation of Tf serum protein concentrations has previously been found in mice (13) and rats (14, 15) exposed to hypobaric hypoxia (0.5 atm) for 1–3 days. Although some of these experiments were established some 40 years ago, the molecular mechanisms leading to hypoxically enhanced Tf expression have not been unraveled so far, mainly due to the lack of a suitable cell culture model.

The hypoxia-inducible factor-1 (HIF-1) was originally iden-

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<sup>1</sup> The abbreviations used are: Tf, transferrin; AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; EMSA, electrophoretic mobility shift assay; Epo, erythropoietin; HBS, HIF-1 binding site; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; bp, base pair(s); kb, kilobase pair(s).

tified by its ability to bind to a hypoxia-responsive *cis*-element located 3' to the Epo gene (16). HIF-1 is a heterodimer consisting of an  $\alpha$  and a  $\beta$  subunit, both belonging to the basic-helix-loop-helix-Per-AhR/ARNT-Sim family of transcription factors (17). Whereas the  $\alpha$  subunit is a novel member of this family, the  $\beta$  subunit is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT) known to heterodimerize with the aryl hydrocarbon receptor/dioxin receptor (AhR) following ligand binding (reviewed in Refs. 7 and 18).

We have established cell culture models to study oxygen-dependent Tf expression and have subsequently analyzed the regulation of the Tf enhancer. Our results demonstrate the presence of two HIF-1 binding sites (HBSs) within the Tf enhancer and show that binding of HIF-1 to these sites confers oxygen-regulated Tf gene expression.

#### MATERIALS AND METHODS

**Cell Culture and Hypoxic Induction**—The human hepatoma cell lines Hep3B and HepG2 were obtained from American Type Culture Collection (ATCC numbers HB-8064 and HB-8065, respectively). The mouse hepatoma cell lines Hepa1 (also termed Hepa1c1c7) and Hepa1C4 (19) were kind gifts of L. Poellinger (Karolinska Institute, Stockholm, Sweden). All cells were cultured in Dulbecco's modified Eagle's medium (high glucose, Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (Boehringer Mannheim), 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin,  $1 \times 10^{-5}$  M non-essential amino acids, 2 mM L-glutamine, and 1 mM sodium pyruvate (all Life Technologies, Inc.) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C. Oxygen tensions in the incubator (Forma Scientific, model 3319) were either 140 mm Hg (20% O<sub>2</sub>, v/v, normoxia) or 7 mm Hg (1% O<sub>2</sub>, v/v, hypoxia). Cells were subjected to hypoxic induction at a cell density of  $2 \times 10^5$  cells/cm<sup>2</sup>. The human epitheloid carcinoma cell line HeLaS3 (ATCC CCL-2.2) was cultured in suspension in Ham's F-12 medium (Life Technologies, Inc.) supplemented as described above. Hypoxic induction was achieved as described elsewhere (20). Briefly, HeLaS3 cells were incubated at a density of  $1 \times 10^7$  cells/ml in an IL 237 tonometer (Instrumentation Laboratory) under continuous stirring for 4 h at 37 °C using gas mixtures of either 20% O<sub>2</sub>, 5% CO<sub>2</sub>, and 75% N<sub>2</sub> (normoxia), or 1% O<sub>2</sub>, 5% CO<sub>2</sub> and 94% N<sub>2</sub> (hypoxia) at a flow rate of 500 ml/min.

**RNA Blot Analysis**—Immediately following stimulation, RNA was isolated as described by Chomczynski and Sacchi (21). Total RNA (10  $\mu$ g) was denatured in formaldehyde and electrophoresed through a 1% agarose gel containing 6% formaldehyde as described (22). Following pressure blotting (Stratagene) to nylon membranes (Biodyne A, Pall) and UV cross-linking (Stratalinker, Stratagene), the filters were hybridized to cDNA probes labeled with [ $\alpha$ -<sup>32</sup>P]dCTP to a specific activity of  $1 \times 10^9$  dpm/ $\mu$ g using the random-primed DNA labeling method (22). Hybridization was performed in 50% formamide, 10% dextran sulfate,  $5 \times$  Denhardt's solution, 200  $\mu$ g/ml sonicated salmon sperm DNA, 1% SDS, 0.9 M NaCl, 60 mM NaH<sub>2</sub>PO<sub>4</sub>, 6 mM EDTA (pH 7.0) for 14 h at 42 °C. The filters were washed to a final stringency of 55 °C in  $0.1 \times$  SSC, 0.2% SDS and the signals recorded using a PhosphorImager (Molecular Dynamics). The Tf,  $\alpha_1$ -antitrypsin,  $\beta$ -actin, ribosomal protein L28, and 28 S ribosomal RNA cDNA probes were obtained as described previously (10, 23). All probes were purified free of vector sequences by restriction digestion and agarose gel purification.

**Plasmid Constructs**—pGLTf4000 was constructed by insertion of the 4-kb *KpnI*-*Bam*HI fragment from the Tf promoter/enhancer-containing plasmid pTfCAT (kindly provided by M. M. Zakin, Institut Pasteur, Paris, France) into the *KpnI*-*Bgl*II sites of pGL3Basic (Promega). Luciferase reporter gene constructs containing the heterologous SV40 promoter were obtained by inserting the DNA sequences of interest into the *Bam*HI site 3' to the luciferase gene of the pGL3Promoter plasmid (Promega). The liver-specific Tf enhancer from nucleotide position -3.6 kb to -3.3 kb relative to the transcriptional start site (3) was obtained by polymerase chain reaction amplification of genomic DNA using the oligonucleotide primers 5'-GGTCAGGCAGAGGACACTG-3' and 5'-CAGTTCTAGACCAACCAAG-3'. The oligonucleotides containing wild type and mutated HBSs are shown in Fig. 4. Copy number and orientation were determined using RVprimer4 (Promega) by T7 polymerase-mediated single-stranded DNA sequencing following the manufacturer's instructions (Pharmacia Biotech Inc.). The  $\beta$ -galactosidase expression vector pCMVlacZ was a kind gift of S. Kozlov (Institute of Biochemistry, Zürich, Switzerland). The HIF-1 $\alpha$  (pCMVhHIF-1 $\alpha$ ) and ARNT/HIF-1 $\beta$  (pCMVhARNT and pCMVhARNT) expression vectors

(19) were generously provided by L. Poellinger.

**Transient Transfections and Reporter Gene Assays**—Hep3B, HepG2, and HeLa cells ( $0.2\text{--}1 \times 10^7$  in 350  $\mu$ l of medium without fetal calf serum) were co-transfected with 25  $\mu$ g each of luciferase and  $\beta$ -galactosidase reporter gene constructs by electroporation at 250 V and 960 microfarads (Gene Pulser, Bio-Rad). After recovering, the cells were split in two aliquots and incubated for 36 h at 20% or 1% O<sub>2</sub>, respectively. After washing twice with phosphate-buffered saline, the cells were lysed in reporter lysis buffer (Promega) and luciferase and  $\beta$ -galactosidase activities were determined according to the manufacturer's instructions (Promega) using a Biocounter M1500 luminometer (Lumac) and a DU-62 spectrophotometer (Beckman), respectively. Differences in the transfection efficiency and extract preparation were corrected by normalization to the corresponding  $\beta$ -galactosidase activities. Luciferase activities were expressed relative to the empty parental vector (pGL3Basic or pGL3Promoter) transfectants. For transient over-expression assays in Hep3B cells, 10  $\mu$ g of each expression vector was co-transfected together with equal amounts of the luciferase reporter construct pTfHBSww and the control plasmid pCMVlacZ. The unrelated vector plasmid pBluescript (Stratagene) was added to adjust the total amount of DNA per electroporation to 50  $\mu$ g.

**Nuclear Extract Preparation**—Nuclear extracts were prepared as described previously (24). Briefly,  $1 \times 10^8$  cells were washed twice with ice-cold phosphate-buffered saline and once with buffer A (10 mM Tris-HCl (pH 7.8), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl). After incubation on ice for 10 min, the cells were lysed by 10 strokes of a Dounce homogenizer, and the nuclei were pelleted and resuspended in buffer C (420 mM KCl, 20 mM Tris-HCl (pH 7.8), 1.5 mM MgCl<sub>2</sub>, 20% glycerol) and incubated at 4 °C for 30 min with gentle agitation. Immediately before use, buffers A and C were supplemented with 0.5 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 2  $\mu$ g/ml each of leupeptin, pepstatin, and aprotinin, and 1 mM Na<sub>3</sub>VO<sub>4</sub> (all obtained from Sigma). The nuclear extract was centrifuged, and the supernatant was dialyzed twice against buffer D (20 mM Tris-HCl (pH 7.8), 100 mM KCl, 0.2 mM EDTA, 20% glycerol). Protein concentrations were determined using the Bradford protein assay (Bio-Rad) with bovine serum albumin as standard.

**Electrophoretic Mobility Shift Assay (EMSA)**—Sequences of the oligonucleotide probes used for EMSA are shown in Fig. 4. The EPOHBS oligonucleotides have been described previously (24). All oligonucleotides (Microsynth) were purified on 10% polyacrylamide gels prior to 5' end-labeling of the sense strand with [ $\gamma$ -<sup>32</sup>P]ATP (Hartmann) using T4-polynucleotide kinase (Fermentas). Unincorporated nucleotides were removed by gel filtration over Bio-Gel P60 (fine) columns (Bio-Rad). Labeled sense strands were annealed to a 2-fold molar excess of unlabeled antisense strands. DNA-protein binding reactions were carried out for 20 min at 4 °C in a total volume of 20  $\mu$ l containing 4–5  $\mu$ g of nuclear extract, 0.1–0.4  $\mu$ g of sonicated, denatured calf thymus DNA (Sigma), and  $1 \times 10^4$  cpm of oligonucleotide probe in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 50 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM EDTA, 5 mM dithiothreitol, and 5% glycerol and run on 4% non-denaturing polyacrylamide gels. Electrophoresis was performed at 200 V in TBE buffer (89 mM Tris, 89 mM boric acid, 5 mM EDTA) at 4 °C, and dried gels were autoradiographed. For supershift analysis, each 1  $\mu$ l of rabbit polyclonal antisera derived against HIF-1 $\alpha$  or ARNT/HIF-1 $\beta$  (kind gift of L. Poellinger) was added to the completed EMSA reaction mixture and incubated for 16 h at 4 °C prior to loading. For competition experiments, a 4–500-fold molar excess of unlabeled annealed oligonucleotides was added to the binding reaction prior to addition of labeled probes.

#### RESULTS

**Oxygen-regulated Tf mRNA Expression in Two Human Hepatoma Cell Lines**—We previously reported on oxygen-regulated mRNA expression of several acute phase genes in the human hepatoma cell line HepG2 (10). Regulation of the Tf gene was of special interest since Tf transcription was down-regulated in response to proinflammatory cytokines (e.g. interleukin-6), but was up-regulated following exposure to low oxygen concentrations. To test whether hypoxic Tf induction observed in HepG2 cells might represent a general phenomenon in liver cells, we also exposed Hep3B cells, another human hepatoma cell line, to 1% O<sub>2</sub> for 1–3 days. As shown by RNA blotting experiments, Tf mRNA was up-regulated about 1.5-fold in Hep3B cells (Fig. 1A) and up to 4.5-fold in HepG2 cells (Fig. 1B). A similar hypoxic induction pattern of endogenous gene expression in the two cell lines was observed for the acute phase reactant  $\alpha_1$ -antitrypsin,



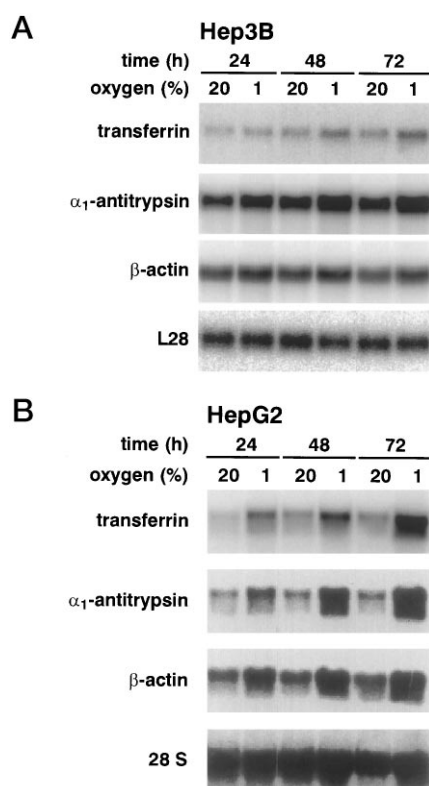


FIG. 1. **RNA blot analysis of hypoxic Tf mRNA induction.** Human Hep3B (A) and HepG2 (B) hepatoma cell lines were exposed to hypoxia as indicated. Hybridization with cDNA probes derived from the ribosomal protein L28 or the ribosomal RNA 28 S served as controls for equal loading and blotting efficiency.

which was included as positive control (Fig. 1, A and B). Specificity of hypoxic up-regulation was shown using L28 and 28 S control hybridizations since  $\beta$ -actin mRNA was also slightly up-regulated in both hepatoma cell lines and thus not suitable as a normalization probe (10).

**The Tf Enhancer Is Hypoxia-responsive**—In a first attempt to identify Tf regulatory sequences conveying hypoxia-inducible Tf transcription, a  $-4000$  to  $+39$  (numbering according to Ref. 3) Tf promoter/enhancer DNA fragment (Fig. 2A) was inserted upstream of a promoterless luciferase reporter gene vector. Following transient transfection into Tf-expressing Hep3B and HepG2 cells, as well as into non-Tf-expressing HeLa cells, this 4-kb Tf promoter/enhancer induced basal luciferase expression 10-, 26-, and 8-fold in Hep3B, HepG2 and HeLa cells, respectively (Fig. 2B, open bars). Hypoxia (1% O<sub>2</sub>) stimulated luciferase expression 4.1- and 5.6-fold in Hep3B and HepG2 cells, respectively, but no significant hypoxic induction could be observed in HeLa cells (Fig. 2B, filled bars). Thus, hypoxia responsiveness seems to be coupled to liver-specific *cis*-acting elements present within this Tf promoter/enhancer DNA fragment.

In analogy to the liver-specific enhancer and the hypoxia-responsive element residing in close vicinity in the Epo 3' flanking region (16), we wondered whether the  $-3600/-3300$  bp liver-specific Tf enhancer (Fig. 2A) might be responsible for oxygen responsiveness of the Tf gene. To test this, we subcloned the 300-bp Tf enhancer downstream of a luciferase reporter gene driven by a heterologous SV40 promoter. The Tf enhancer induced normoxic luciferase expression by 8.1-, 1.6-, and 2.0-fold in Hep3B, HepG2, and HeLa cells, respectively (Fig. 3, open bars). This expression level was further up-regulated by exposing the cells to hypoxia; luciferase activity in Hep3B, HepG2, and HeLa cells increased 3.1-, 6.8-, and 1.8-fold, re-

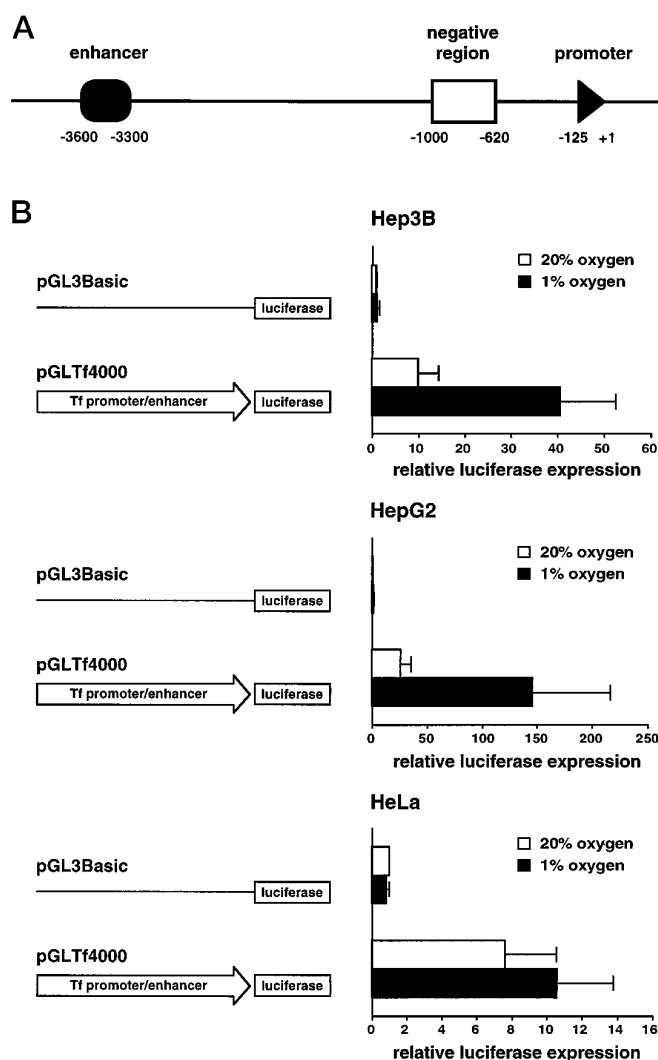


FIG. 2. **Hypoxia responsiveness of the Tf 5' flanking region.** A, structural organization of the 5' flanking region of the Tf gene (according to Ref. 4). B, luciferase reporter gene activity following transient transfection and hypoxic induction for 36 h of Hep3B, HepG2, and HeLa cells with luciferase expression plasmids containing the 4-kb Tf promoter/enhancer. A co-transfected  $\beta$ -galactosidase expression vector served as internal control for transfection efficiency and extract preparation. All values were normalized to the normoxic luciferase activities obtained with the empty pGL3Basic vector which were arbitrarily defined as 1. Means  $\pm$  S.D. of three to four independent experiments are shown.

spectively (Fig. 3, filled bars). The weak hypoxic inducibility (1.4-fold) of the pGL3Promoter plasmid itself has been reported previously (24). Thus, similar to the observations using the 4-kb Tf promoter/enhancer, the 300-bp Tf enhancer alone conferred hypoxia inducibility in Hep3B and HepG2 hepatoma cells, but was not significantly active in non-Tf-expressing HeLa cells.

**Two Tandemly Repeated HBSs Confer Hypoxia Responsiveness to the Tf Gene**—A computer-assisted search using a HIF-1 consensus DNA-binding site (24) as query revealed the presence of two tandemly arrayed putative HBSs beginning at nucleotide positions 174 and 191, respectively (Fig. 4), within the 300-bp Tf enhancer (numbering according to Ref. 3). No other matches to the HIF-1 query were found in the published nucleotide sequences of the Tf gene. To test whether these two putative HBSs were functionally oxygen-responsive, we synthesized oligonucleotides containing both sites in either the wild type configuration (TfHBSww), or with one (TfHBSw or

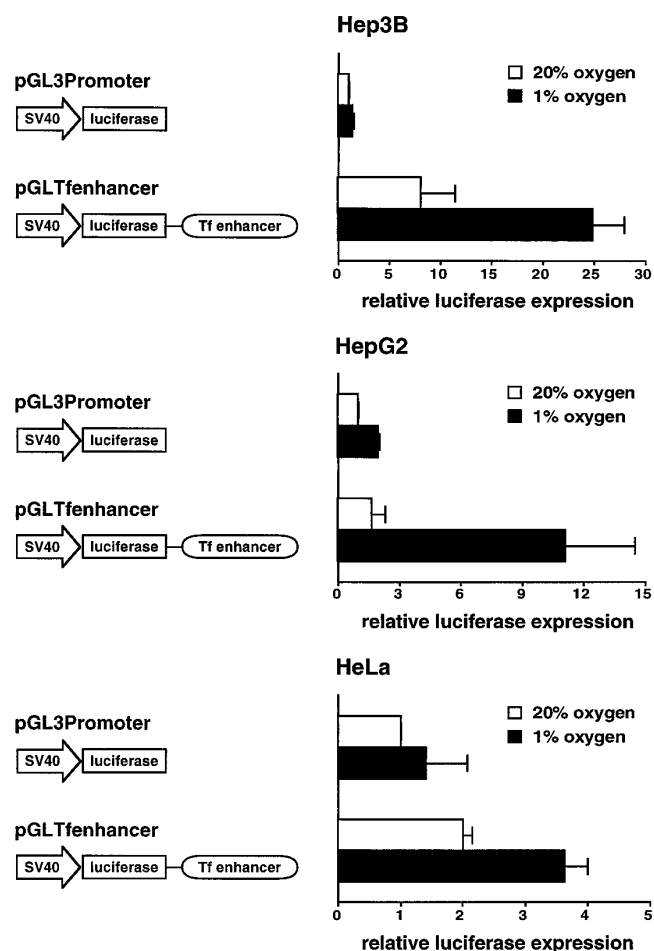


FIG. 3. **Hypoxia responsiveness of the Tf enhancer.** SV40 promoter-driven luciferase constructs with or without the 300-bp Tf enhancer (–3600 to –3300) were transiently transfected into Hep3B, HepG2 and HeLa cells. After 36 h of normoxic or hypoxic culture, luciferase activities were determined as described in Fig. 2. Means  $\pm$  S.D. of three independent experiments are shown.

NAME	SEQUENCE
EPOHBS	3082 tgagac AGCACGTA gggc 3065 3082 tgagac AGCACGTA gggc 3065
TfHBSww	170 ttcc TGCACGTA cacacaaag CGCACGTA ttcc 201
TfHBSwm	170 ttcc TGCACGTA cacacaaag CGCt <del>ttt</del> TA ttcc 201
TfHBSmw	170 ttcc TGCt <del>ttt</del> TA cacacaaag CGCACGTA ttcc 201
TfHBSmm	170 ttcc TGCt <del>ttt</del> TA cacacaaag CGCt <del>ttt</del> TA ttcc 201

FIG. 4. **Oligonucleotides carrying wild type (w) and mutant (m) HBSs derived from the Epo (24) and Tf genes (3).** Note that for better comparison the complementary strand of EPOHBS is shown twice.

TfHBSmw) or both (TfHBSmm) HBS sites mutated (Fig. 4). Single copies of these oligonucleotides were inserted 3' to a luciferase reporter gene driven by a heterologous SV40 promoter. For comparison, a hypoxia-responsive luciferase construct (pGLEPOHBS.3) containing three concatamerized copies of the Epo HBS was included in this study (24). Luciferase activity was determined following transient transfection of Hep3B and HeLa cells, splitting in two aliquots and 36 h of normoxic or hypoxic cell culture. Compared with the normoxic control cells, hypoxia increased luciferase expression from the control plasmid (pGLEPOHBS.3) 4.1- and 6.8-fold in Hep3B and HeLa cells, respectively (Fig. 5). Hypoxic induction medi-

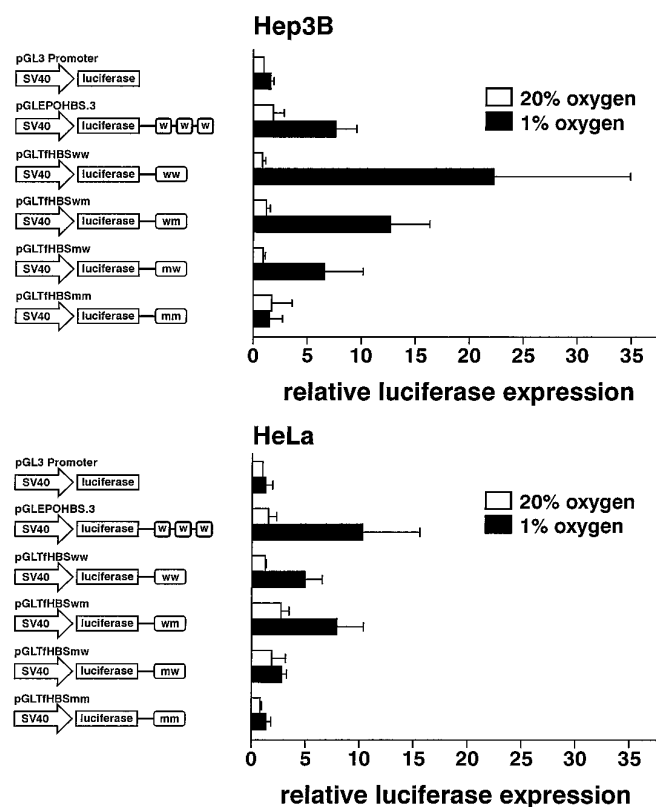


FIG. 5. **The Tf enhancer contains two functional HBSs.** Luciferase reporter gene constructs containing three copies of the EPOHBS (W) or a single copy of the two tandemly arrayed putative HBSs derived from the Tf enhancer (see Fig. 4) were transiently transfected into Hep3B and HeLa cells. Following normoxic or hypoxic exposure for 36 h, luciferase activity was determined as described in Fig. 2. Means  $\pm$  S.D. of 3–10 independent experiments are shown.

ated by the two tandemly arrayed, putative Tf HBSs (pGLTfHBSww) was more effective in Hep3B cells (9.4-fold) than in HeLa cells (3.7-fold). Although again less pronounced in HeLa cells compared with Hep3B cells (similar to the 300-bp Tf enhancer; see Fig. 3), the putative Tf HBSs functioned as hypoxia-dependent enhancer in both cell lines that do or do not express Tf (Fig. 5). This observation is reminiscent of the Epo HBS, which has previously been reported to enhance hypoxic gene expression in Epo-expressing and non-Epo-expressing cell lines (25, 26). Mutation of either one of the two putative Tf HBSs (plasmids pGLTfHBSwm and pGLTfHBSmw) only partially reduced hypoxic luciferase expression, and a double mutation of both sites (plasmid pGLTfHBSmm) was necessary to completely abrogate oxygen responsiveness down to the basal level observed with the empty vector alone (Fig. 5).

**HIF-1 Activates Reporter Gene Expression via the Tf HBS**—To investigate the involvement of the HIF-1 protein complex in Tf regulation, we performed transient expression experiments using the HIF-1 $\alpha$  and/or ARNT/HIF-1 $\beta$  expression vectors pCMVhHIF-1 $\alpha$  and pCMVhARNT, respectively (19). They were co-transfected into Hep3B cells together with the reporter gene construct pGLTfHBSww (depicted in Fig. 5), the normalization plasmid pCMVlacZ and the unrelated plasmid pBluescript used to equalize the total amount of DNA per transfection. As shown in Fig. 6 (open bars), transient overexpression, under normoxic conditions, of either of the two HIF-1 subunits weakly (about 2-fold) induced reporter gene expression, whereas expression of both HIF-1 subunits induced luciferase expression by 5.8-fold (Fig. 6, open bars). Co-expression with a reporter gene construct containing mutant HBSs (pGLTfHBSmm) did not result in enhanced luciferase expres-

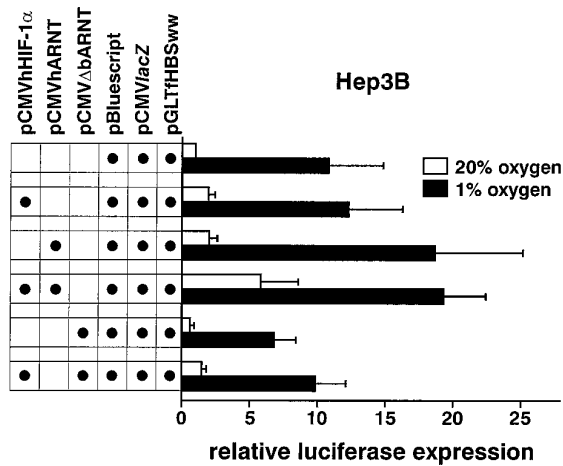


FIG. 6. HIF-1 subunits transactivate Tf HBS-containing reporter gene constructs. Hep3B cells were transiently co-transfected with HIF-1 $\alpha$  and ARNT/HIF-1 $\beta$  expression vectors together with the Tf HBS-containing luciferase reporter gene construct pGLTHBSsw, the control vector pCMVlacZ, and the unrelated plasmid pBluescript to equalize the total amount of DNA per transfection. Following normoxic or hypoxic exposure, luciferase activity was determined, corrected for transfection efficiency and extract preparation yield according to the  $\beta$ -galactosidase activity, and normalized to the normoxic value of pGLTHBSsw control transfection, which was arbitrarily defined as 1. Means  $\pm$  S.D. of three independent experiments are shown.

sion (data not shown), implying that HIF-1 needs to bind to the Tf HBSs to transactivate reporter gene expression. Hypoxia also activated the Tf HBSs (Fig. 6, filled bars), and overexpression of the two HIF-1 subunits further enhanced this effect 1.8-fold. Interestingly, overexpression of a dominant negative ARNT/HIF-1 $\beta$  mutant (pCMV $\Delta$ hARNT), which lacks the basic domain and hence is still capable of heterodimerizing with HIF-1 $\alpha$  but cannot bind DNA (19), reduced reporter gene expression by sequestering endogenous as well as overexpressed HIF-1 $\alpha$  (Fig. 6), underlining the critical role of HIF-1 in hypoxic Tf induction.

**DNA Binding of HIF-1 to the Two HBS Sites of the Tf Enhancer**—To directly identify the endogenous transcription factor(s) binding to the HBS of the Tf enhancer, EMSAs were performed using the TfHBS oligonucleotides shown in Fig. 4 as probes. Following incubation of the TfHBSsw probe with nuclear extracts derived from normoxic or hypoxic Hep3B cells, nonspecific, constitutive, and hypoxia-inducible factors were detected (Fig. 7A). Using Hep3B nuclear extracts, mutation of the 3' HBS site (oligonucleotide TfHBSwm) did not greatly affect binding of the hypoxia-inducible factor. In contrast, mutation of the 5' HBS site present in the Tf enhancer (oligonucleotide TfHBSmw) strongly reduced but (as could be seen after prolonged exposure, data not shown) did not completely abolish protein binding. Only the double mutation (oligonucleotide TfHBSmm) completely abolished binding of both the hypoxia-inducible and the constitutive factor, indicating that both sites are capable of binding these two factors although with different affinities. Interestingly, in Hep3B cells, constitutive factor binding was found only with single mutations but not with the wild type or double mutant probe. To date, we have no explanation for this observation. In the case of the Epo HBS, we previously identified the constitutive factor as ATF-1/CREB-1 family members contacting similar nucleotide residues as HIF-1 itself (24). Similar constitutive and hypoxia-inducible factors derived from the non-Tf-expressing human HeLa cervical carcinoma and mouse L929 fibroblast cells, as well as from the Tf-expressing mouse Hepa1 hepatoma cells, also bound to the TfHBSsw probe (Fig. 7B). In contrast to Hep3B cells, constitutive factor binding to the TfHBSsw probe was detect-

able in all of these extracts.

To demonstrate that the hypoxia-inducible factor binding the TfHBSsw probe is indeed identical with the previously identified HIF-1 (17), nuclear extracts were prepared from normoxic and hypoxic Hepa1C4 cells and analyzed by EMSA. The cell line Hepa1C4, a subline of Hepa1 cells, is deficient in functional ARNT/HIF-1 $\beta$  expression (19) and devoid of DNA binding activity to the EPOHBS probe as well as of reporter gene induction with Epo HBS luciferase constructs (19). As shown in Fig. 7B, this cell line also lacked hypoxia-inducible TfHBSsw DNA binding activity, whereas the constitutive and nonspecific factors were still present. Moreover, rabbit polyclonal antibodies derived against HIF-1 $\alpha$  and ARNT/HIF-1 $\beta$  supershifted the hypoxia-inducible factor binding to the EPOHBS probe as well as to the TfHBS wild type and single mutated probes in Hepa1 cells (Fig. 7C), suggesting that this factor is functionally and immunologically indistinguishable from HIF-1.

To analyze in more detail the binding of HIF-1 to the two functional Tf HBSs, competition experiments were performed using the labeled TfHBSsw probe and increasing amounts of unlabeled wild type or mutant oligonucleotides (depicted in Fig. 4). As shown in Fig. 8, using nuclear extracts derived from normoxic or hypoxic Hepa1 cells, the 3' single mutant oligonucleotide (TfHBSwm) competed as efficiently for HIF-1 DNA binding to the TfHBSsw probe as the wild type TfHBSsw oligonucleotide itself (*i.e.* competition was observed using a 20-fold molar excess). The reduction in HIF-1 band intensity with 20- and 100-fold molar excesses of the 5' single mutant oligonucleotide (TfHBSmw) was somewhat less prominent, confirming that although both Tf HBSs can bind HIF-1, the 3' site has a lower affinity for HIF-1. The double mutant TfHBSmm oligonucleotide did not compete for HIF-1 DNA binding activity (Fig. 8). On the other hand, oligonucleotide EPOHBS also competed for HIF-1 DNA binding to the TfHBSsw probe, although with a lower efficiency than TfHBSsw itself (*i.e.* more than a 100-fold molar excess was necessary for competition), probably due to the presence of two HBSs on the TfHBSsw oligonucleotide compared with only one HBS in the EPOHBS oligonucleotide.

**Tf mRNA Is Not Induced in ARNT/HIF-1 $\beta$ -deficient Hepa1C4 Cells**—To investigate whether HIF-1 is capable of hypoxically inducing the endogenous Tf gene, we made use of the ARNT/HIF-1 $\beta$ -deficient Hepa1C4 cell line, which was cultured at 20% or 1% O<sub>2</sub> and analyzed by RNA blotting and hybridization. In previous experiments, we have demonstrated a lack of hypoxic aldolase mRNA induction and a reduction in hypoxic VEGF mRNA induction in this cell line (19). Whereas in the parental ARNT/HIF-1 $\beta$ -positive Hepa1 cell line hypoxia reproducibly induced Tf mRNA by a factor of 1.5 over the normoxic control, Hepa1C4 cells did not show induction of Tf mRNA (Fig. 9). This is in agreement with the lack of HIF-1 DNA-binding activity to the TfHBSsw probe in these cells (shown in Fig. 6B) and, despite the rather low Tf mRNA expression levels and hypoxic inducibility in this particular hepatoma cell line, confirms that HIF-1 is critically involved in the oxygen responsiveness of the Tf gene.

## DISCUSSION

**Tf Is a New Member of the HIF-1-regulated Gene Family**—HIF-1 was originally defined by its capability of binding to a site required for hypoxic induction of Epo gene transcription (16, 17). Other examples of HIF-1-dependent oxygen-regulated genes include those encoding for glycolytic enzymes (27–30), vascular endothelial growth factor (VEGF, Refs. 31–33), inducible nitric oxide synthase (34), and glucose transporter-1 (Glut-1, Ref. 35). We have previously found that Tf gene expression is hypoxically induced in HepG2 hepatoma cells (10).



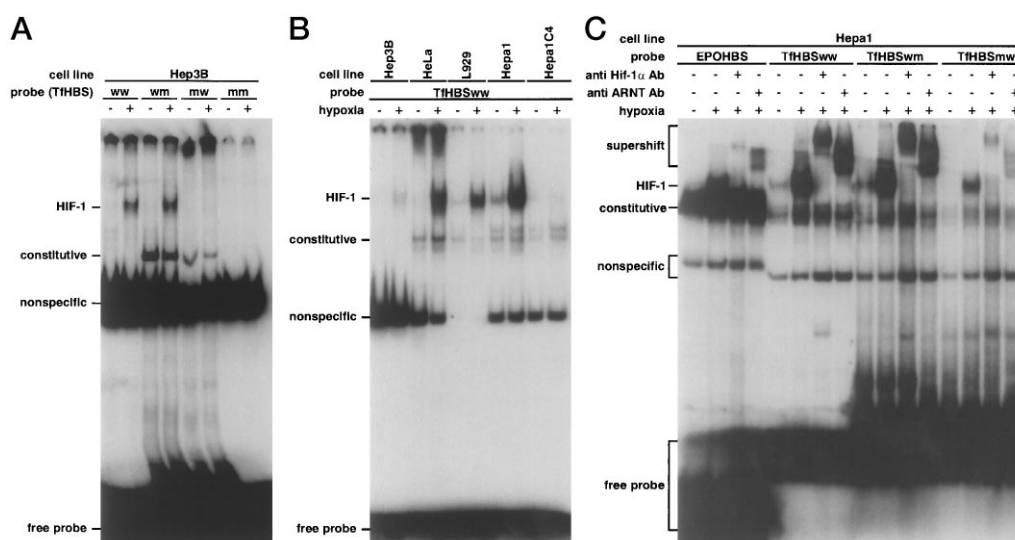


FIG. 7. **HIF-1 binds to the Tf HBSs.** A, HIF-1 DNA binding activity was assessed by EMSA using the oligonucleotide probes described in Fig. 4 and nuclear extracts derived from Hep3B cells cultured at normoxic or hypoxic conditions for 4 h. B, widespread hypoxia-inducible DNA binding activity to the wild type TfHBSww probe in Tf expressing (Hep3B and Hepa1) and non-expressing cell lines (HeLa and L929). Hepa1C4 is a subline of the mouse hepatoma Hepa1, lacking functional ARNT/HIF-1 $\beta$ . C, supershift analysis of HIF-1 bound to the TfHBS probes or to the EPOHBS probe using polyclonal rabbit antibodies derived against HIF-1 $\alpha$  and ARNT/HIF-1 $\beta$ .

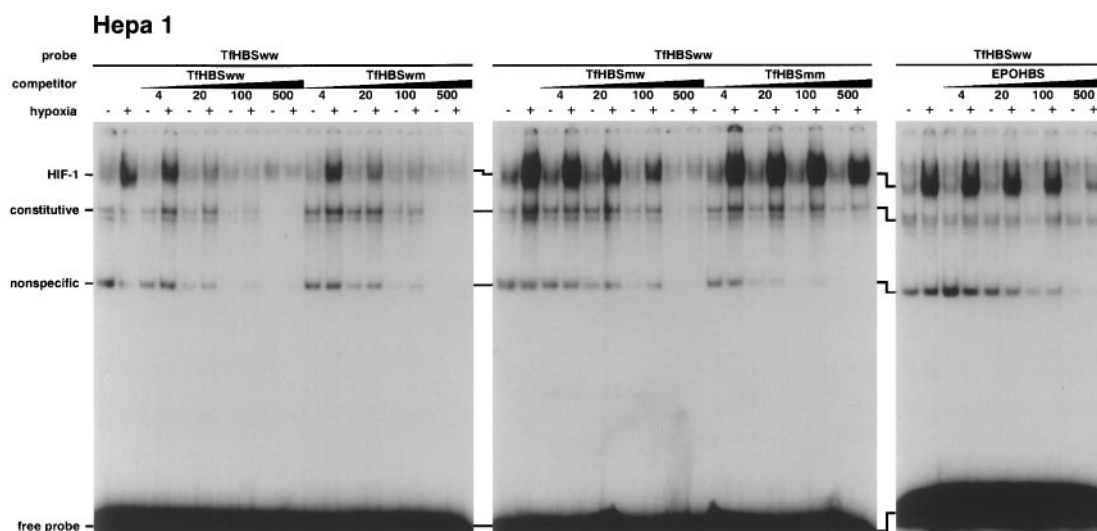


FIG. 8. **Both Tf HBS sites are capable of HIF-1 DNA binding.** EMSA competition experiments using an increasing molar excess of unlabeled oligonucleotide (Fig. 4) as indicated. Nuclear extracts were derived from Hepa1 cells cultured at 20% or 1% O<sub>2</sub> for 4 h.

In this paper, we demonstrate that this effect is also mediated by HIF-1 via binding of two HBSs present in the Tf enhancer. We and others recently characterized a previously obtained mouse hepatoma cell line (Hepa1C4), which lacks functional ARNT expression, and demonstrated that it is also devoid of functional HIF-1 expression in terms of DNA binding activity and reporter gene transactivation (19, 33, 36, 37). By using these Hepa1C4 cells, we found that ARNT/HIF-1 $\beta$  is necessary for formation of the hypoxia-inducible complex binding to the Tf HBSs. Moreover, hypoxic induction of the endogenous Tf gene is also abrogated in Hepa1C4 cells, suggesting that HIF-1 is critically involved in oxygen-regulated Tf gene expression.

**Architecture of the Tf HBS**—An interesting feature of the Tf enhancer is the presence of two functional HBSs in close vicinity to each other. Such an architecture was not found in the Epo 3' HBS. The finding that a single HBS derived from the Epo HBS was not sufficient to convey oxygen responsiveness to a heterologous promoter driving expression of a reporter gene, although the HIF-1 complex bound to this site *in vitro* (24), raised the question as to whether additional functional *cis*-

acting elements are necessary for full activation. Such an additional element (CACA) was found close to the HBSs in the Epo (16) and VEGF genes (33), as well as in the genes encoding several glycolytic enzymes (30). The transacting factors binding to this element, however, still remain to be identified. As suggested by the hypoxic induction of reporter gene expression by concatamerized HBSs, such an additional element could also be the HIF-1 site itself (Refs. 24 and 27 and this report). In addition, an activator protein (AP-1) site in the vicinity of the HIF-1-binding HBS in the 5' flanking region of the VEGF gene (31) and a cAMP-responsive element close to the two consensus HBSs in the LDH gene (28) have been implicated in full hypoxic induction of gene expression. Protein-DNA interactions of cAMP-responsive element-binding transcription factors (ATF-1 and CREB-1) were also observed within the HIF-1 site of the Epo HBS itself (24). In conclusion, it seems to be a common feature of an HBS that a single HBS in isolation is not sufficient to convey full hypoxic activation of gene expression. The additional factors required for full activation, however, might differ between the oxygen-regulated genes.

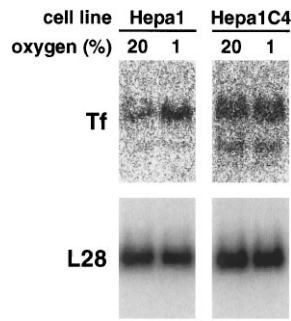


FIG. 9. **HIF-1 is critically involved in hypoxic induction of the endogenous Tf gene.** RNA blot analysis of ARNT/HIF-1 $\beta$ <sup>+</sup> Hepa1 and ARNT/HIF-1 $\beta$ <sup>-</sup> Hepa1C4 cells cultured at normoxia or hypoxia for 16 h. Hybridization to a cDNA probe derived from the ribosomal protein L28 served as a control for equal loading and blotting efficiency.

**Protein-DNA Interactions at the Tf HBS**—As mentioned above, the hypoxia-inducible Tf enhancer region is composed of two adjacent HBSs, both of which are capable of conveying hypoxic induction to reporter gene expression. The two 8-bp HBSs in the Tf enhancer are spaced by 9 bp only (Fig. 4), raising the question whether two HIF-1 complexes could bind simultaneously to these two sites or whether due to sterical hindrance only one complex can bind at once. As shown by the equal migration properties of TfHBS (wild type and single mutant) and EPO HBS probes in our EMSAs, the predominant protein-DNA complex might consist of only one HIF-1 heterodimer bound to the TfHBS oligonucleotide. Although EMSA analysis is probably not the ideal method to determine molecular mass differences, we would predict that an increase of more than 200 kDa (the molecular mass of an additional HIF-1 heterodimer) should be detectable.

The two core sequences TACGTGCA and TACGTGCG (note that the complementary strand to that shown in Fig. 4 is given) conform well with a previously published (33) consensus HBS (BACGTGSK, where B is C or G or T, S is C or G, and K is G or T). Strikingly, the presence of an adenosine residue at position 8 of the 8-bp consensus sequence (found in the 5' TfHBS) has never been reported in any of the so far published genes carrying HBSs (18). Moreover, positions 9 and 10 also did not contain an adenosine residue in these HBSs (not shown). Thus, one would predict that this lack of adenosine residues in the 3' part of the HBS is of functional relevance and that the DNA binding affinity of HIF-1 for the 5' Tf HBS might be decreased. Surprisingly, as shown with mutated oligonucleotide probes and competition experiments, the 3' rather than the 5' HIF-1 site (which contains the unexpected adenosine residue) constantly produced lower HIF-1 band intensities in EMSAs. On the functional level, only mutation of the 5' Tf HBS significantly reduced (but did not completely abolish) hypoxic induction of reporter gene expression, confirming that the 5' HBS is more effective in HIF-1 binding than the 3' HBS. Thus, despite the considerable number of so far identified HBSs, a conclusive consensus sequence still remains to be determined.

**How Is HIF-1 Itself Activated?**—Having established that HIF-1 is mediating hypoxic induction of Tf gene expression, the question arises of how HIF-1 itself is activated. So far, little is known about the mechanisms of oxygen sensing and subsequent conditional regulation of HIF-1. Initially, it has been reported that HIF-1 $\alpha$  (and to a lesser extent also ARNT/HIF-1 $\beta$ ) is regulated at the level of mRNA expression (17), but work from our and other laboratories could not confirm this result (19, 38, 39). Hence, HIF-1 must be regulated at the post mRNA level. Possible mechanisms include translational up-regulation, post-translational protein stabilization (39) or protein modifications such as phosphorylation (40) or redox modifica-

tions (41). There is good evidence for all of these putative mechanisms and more than one might turn out to be involved in hypoxic HIF-1 activation (reviewed in Ref. 18). Interestingly, our transient overexpression experiments using the Tf HBS demonstrated that forced expression of the two HIF-1 subunits is sufficient to convey induction to reporter gene transcription even under normoxic conditions. This observation is in agreement with recent work using the Epo HBS (19, 42) and VEGF HBS (33). Since conditional regulation thus does not seem to be of primary importance for HIF-1 function, we favor the model(s) of translational up-regulation and/or protein stabilization. However, hypoxia represented a stronger stimulus than overexpression of HIF-1, indicating that for a full response conditional regulation of HIF-1 is required. Further investigations of HIF-1 regulation is crucial for the elucidation of the signal transduction pathway(s) involved in the expression of Tf as well as other oxygen-regulated genes.

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## REFERENCES

- Bonkovsky, H. L. (1991) *Am. J. Med. Sci.* **301**, 32–43
- Finch, C. (1994) *Blood* **84**, 1697–1702
- Boissier, F., Augé-Gouillou, C., Schaeffer, E., and Zakin, M. M. (1991) *J. Biol. Chem.* **266**, 9822–9828
- Sawaya, B. E., Aunis, D., and Schaeffer, E. (1996) *J. Neurosci. Res.* **43**, 261–272
- Augé-Gouillou, C., Petropoulos, I., and Zakin, M. M. (1993) *FEBS Lett.* **323**, 4–10
- Petropoulos, I., Augé-Gouillou, C., and Zakin, M. M. (1991) *J. Biol. Chem.* **266**, 24220–24225
- Bunn, H. F., and Poyton, R. O. (1996) *Physiol. Rev.* **76**, 839–885
- Jelkmann, W. (1992) *Physiol. Rev.* **72**, 449–489
- Goldberg, M. A., Glass, G. A., Cunningham, J. M., and Bunn, H. F. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 7972–7976
- Wenger, R. H., Rolfs, A., Marti, H. H., Bauer, C., and Gassmann, M. (1995) *J. Biol. Chem.* **270**, 27865–27870
- Heinrich, P. C., Castell, J. V., and Andus, T. (1990) *Biochem. J.* **165**, 621–636
- Mendel, G. A. (1961) *Blood* **18**, 727–736
- Simpson, R. J. (1992) *Ann. Hematol.* **65**, 260–264
- Colehour, J. K., Borsook, H., and Graybiel, A. (1957) *Am. J. Physiol.* **191**, 113–114
- Osterloh, K. R. S., Simpson, R. J., Snape, S., and Peters, T. J. (1987) *Blut* **55**, 421–431
- Semenza, G. L., and Wang, G. L. (1992) *Mol. Cell. Biol.* **12**, 5447–5454
- Wang, G. L., Jiang, B.-H., Rue, E. A., and Semenza, G. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 5510–5514
- Wenger, R. H., and Gassmann, M. (1997) *Biol. Chem.* **378**, 609–616
- Gradin, K., McGuire, J., Wenger, R. H., Kvietikova, I., Whitelaw, M. L., Toffgård, R., Tora, L., Gassmann, M., and Poellinger, L. (1996) *Mol. Cell. Biol.* **16**, 5221–5231
- Jiang, B.-H., Semenza, G. L., Bauer, C., and Marti, H. H. (1996) *Am. J. Physiol.* **271**, C1172–C1180
- Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Wenger, R. H., Marti, H. H., Schuerer-Maly, C. C., Kvietikova, I., Bauer, C., Gassmann, M., and Maly, F. E. (1996) *Blood* **87**, 756–761
- Kvietikova, I., Wenger, R. H., Marti, H. H., and Gassmann, M. (1995) *Nucleic Acids Res.* **23**, 4542–4550
- Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2423–2427
- Wang, G. L., and Semenza, G. L. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 4304–4308
- Firth, J. D., Ebert, B. L., Pugh, C. W., and Ratcliffe, P. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 6496–6500
- Firth, J. D., Ebert, B. L., and Ratcliffe, P. J. (1995) *J. Biol. Chem.* **270**, 21021–21027
- Semenza, G. L., Roth, P. H., Fang, H.-M., and Wang, G. L. (1994) *J. Biol. Chem.* **269**, 23757–23763
- Semenza, G. L., Jiang, B.-H., Leung, S. W., Passantino, R., Concordet, J.-P., Maire, P., and Giallongo, A. (1996) *J. Biol. Chem.* **271**, 32529–32537
- Levy, A. P., Levy, N. S., Wegner, S., and Goldberg, M. A. (1995) *J. Biol. Chem.* **270**, 13333–13340
- Liu, Y., Cox, S. R., Morita, T., and Kourembanas, S. (1995) *Circ. Res.* **77**, 638–643
- Forsythe, J. A., Jiang, B.-H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D., and Semenza, G. L. (1996) *Mol. Cell. Biol.* **16**, 4604–4613
- Melillo, G., Musso, T., Sica, A., Taylor, L. S., Cox, G. W., and Varesio, L. (1995) *J. Exp. Med.* **182**, 1683–1693



35. Ebert, B. L., Firth, J. D., and Ratcliffe, P. J. (1995) *J. Biol. Chem.* **270**, 29083–29089
36. Wood, S. M., Gleadle, J. M., Pugh, C. W., Hankinson, O., and Ratcliffe, P. J. (1996) *J. Biol. Chem.* **271**, 15117–15123
37. Li, H., Ko, H. P., and Whitlock, J. P., Jr. (1996) *J. Biol. Chem.* **271**, 21262–21267
38. Wenger, R. H., Rolfs, A., Marti, H. H., Guénet, J.-L., and Gassmann, M. (1996) *Biochem. Biophys. Res. Commun.* **223**, 54–59
39. Huang, L. E., Arany, Z., Livingston, D. M., and Bunn, H. F. (1996) *J. Biol. Chem.* **271**, 32253–32259
40. Wang, G. L., Jiang, B.-H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* **216**, 669–675
41. Wang, G. L., Jiang, B.-H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* **212**, 550–556
42. Jiang, B.-H., Rue, E., Wang, G. L., Roe, R., and Semenza, G. L. (1996) *J. Biol. Chem.* **271**, 17771–17778